DICT 2116

IN THE UNITED STATES DISTRICT COURT
FOR THE NORTHERN DISTRICT OF OKLAHOMA

STATE OF OKLAHOMA,	)	
	)	
Plaintiff,	)	
	)	
v.	)	**Case No. 05-cv-329-GKF(PJC)**
	)	
TYSON FOODS, INC., et al.,	)	
	)	
Defendants.	)	

DECLARATION OF Tamzen Macbeth Ph.D., P.E.

I, Tamzen Macbeth Ph.D., P.E hereby declare as follows:

1. My education includes a Ph.D. from the University of Idaho, 2008, a M.S. in Environmental Engineering from the Idaho State University, 2002, and a B.S. in Microbiology, from the Idaho State University, 2000. I received training in conducting molecular DNA and RNA analysis as part of my education in microbiology at Idaho State University, and while conducting graduate work under a graduate fellowship program at the Department of Energy's Idaho National Laboratory. In addition, I was responsible for molecular laboratory work at North Wind Inc., in collaboration with Idaho State University, from 2004 to date. I have over nine years of experience using molecular biological tools to evaluate microbiological communities in the environment. I have been trained and have expertise conducting molecular analyses including analyses conducted as part of the poultry biomarker in this case, namely polymerase chain reaction (PCR), quantitative PCR (qPCR), denaturing gradient gel electrophoresis (DGGE), terminal

Courts Exhibit #1

PLAINTIFF'S EXHIBIT 116

restriction fragment length polymorphism (T-RFLP), clone libraries, DNA sequencing, and DNA sequence analysis including alignment, and phylogenetics, amongst others, for identification and characterization of microorganisms. I specialize in evaluating a variety of environmental media including soils, groundwater, surface water, and fecal material using molecular biological tools to identify microorganisms of interest, and track their spatial and/or temporal distribution, evaluate population dynamics in response to changes in environmental conditions, and evaluate fate and transport of microorganisms of interest within the environment.

2. I have reviewed the expert report submitted by Dr. Valerie Harwood and the Defendants' motion to exclude her expert testimony. I have also reviewed the expert report and the considered materials submitted by the Defendants' expert witness Dr. Samuel Myoda. I also attended the deposition of Dr. Myoda.

3. I initially became involved in the work of Dr. Harwood in the IRW case as a result of my affiliation with Dr. Kent Sorenson of CDM, who I worked with at the DOE Idaho National Laboratory developing molecular assays to evaluate microorganisms. Dr. Sorenson was aware of my experience, capabilities and interest in pursuing the development of molecular microbiological assays to identify, characterize, and evaluate important microorganisms within different environments. As it was explained to me, the objective of the project was to determine if we could use molecular biological tools to identify a unique microbial signature for poultry litter that could be used to determine if poultry litter was present in environmental samples collected from the IRW.

4. Our first task to develop a poultry biomarker was to identify the appropriate microbial source tracking (MST) suite of tools that were already published and accepted within the scientific community. Those identified included PCR, T-RFLP, clone library, DNA sequence analysis and quantitative PCR. The results of our initial testing demonstrated the poultry biomarker qPCR assay could be used reliably within the IRW to evaluate the presence of poultry litter within environmental samples. Once we had developed a poultry-specific biomarker applicable to the IRW, North Wind, with the review of Dr. Harwood, generated a standard operating procedure detailing the optimized methods for the poultry biomarker assay. Samples collected from the IRW were analyzed in the North Wind laboratory for the poultry biomarker using the SOP for the qPCR protocol. We analyzed the samples and provided data reports on results to Dr. Harwood who used these results in her expert report.

5. Subsequent to this initial testing, we have expanded the specificity testing of the poultry biomarker to geographic regions outside the IRW to include additional poultry feces, fecal swabs, and litter (collected from Georgia, Florida, Minnesota, Utah); geese (collected from Minnesota and Idaho); ducks (collected from Idaho); beef cattle (collected from Minnesota); and WWTP influent and effluent from samples across the United States. This testing has demonstrated that not only is the poultry biomarker 100% sensitive and specific within the IRW, it is 100% sensitive to poultry broiler litter and feces, 80% sensitive to poultry litter and broiler and layer feces and fecal swabs, and 93% specific when challenged against 116 non-target fecal samples representing a much larger geographic region.

6. The poultry biomarker assay was also independently validated in Dr. Michael Sadowsky's laboratory at the University of Minnesota. I was involved in reviewing the approach and providing consulting on developing and optimizing the poultry biomarker qPCR assay at Dr. Sadowsky's lab. Blind samples including 13 soiled poultry litter samples from four different facilities, 31 composite, non-target fecal samples (10 beef cattle, 3 dairy cattle, 2 swine, 5 geese, 5 duck, and 6 raw human sewage), four soil samples from fields on which poultry litter had been spread, and five edge of field runoff water samples were analyzed by the Sadowsky/University of Minnesota lab. Dr. Sadowsky's blind test confirmed the reliability of the poultry biomarker.

7. The approach for developing a poultry-specific biomarker was based on methods characterized by the EPA in their publications "Microbial Source Tracking Guide Document (2005)", and "Detecting and Mitigating the Environmental Impact of Fecal Pathogens Originating from Confined Animal Feeding Operations: Review" as "library-independent techniques"[1, 2]. These "library-independent techniques" are also characterized as microbial molecular techniques, which have been developed as a result of the recognition that as few as 0.1-10% of microorganisms can be enriched through standard culturing practices. The ability to evaluate microbial communities much closer to their natural state has led to significant developments in identifying organisms. The premise behind using molecular tools, or library-independent techniques, to identify microbial populations unique to a specific source of fecal waste, is that the probability of identifying a unique microorganism is much greater using these tools because they provide a much more comprehensive view of microbial community diversity. These

techniques are much more reproducible, specific and robust than other methods for MST. These methods have greatly expanded the ability to:

1) identify the diversity of microorganisms within various environmental compartments (i.e. groundwater, soils, surface water),

2) identify microorganisms of interest within a particular environmental sample (i.e. poultry litter), and

3) compare the relative presence and abundance of these micoroorganisms within different samples (i.e. different sources of feces), a given spatial area (i.e. the IRW) or temporally over time (i.e. seasonally) to understand their sources, and distribution within the environment.

8. The development and extensive availability of molecular methods have resulted in methods to effectively and reproducibly isolate DNA from environmental samples without need for cultivation. For instance, the vast majority of the "library-independent" techniques rely on replication of DNA using polymerase chain reaction (PCR), which was the focus of our methods used during the poultry biomarker development. PCR relies on using DNA primers to target a region of DNA and replicate that DNA "target." The utility of PCR is also greatly enhanced by quantitative PCR (qPCR). One of the limitations of PCR is that it only measures the end point of the assay and so interpretation is limited to whether or not the sample was positive (DNA was amplified by the primers) or negative (DNA was not amplified by the primers). qPCR simultaneously amplifies and quantifies the amount of DNA that is being replicated with each PCR cycle. This can be used to generate standard curves of known amounts of the DNA target, much like

analytical chemistry, to determine a relative quantity of the target DNA sequence present in the unknown sample that is being analyzed. In addition, certain qPCR applications, such as those using SYBR green chemistry, have the added advantage of conducting melt curve analysis in order to verify the efficiency and specificity of the qPCR reaction. For instance, the melt curve analysis can be used to determine if one DNA sequence or multiple DNA sequences were amplified in the qPCR reaction. This is particularly important for MST applications such as the poultry biomarker where it is important to ensure the specificity of your reaction (i.e. you are amplifying your intended biomarker and not some other DNA sequence).

9. PCR and qPCR have been widely accepted and used because of their sensitivity, accuracy and precision; an extensive knowledge base regarding the development, optimization, and standardization of the assays; and the availability of standardized instrumentation, reagent mixes and analytical software, including software to efficiently design PCR primers. PCR-based techniques have been used widely in the fields of molecular biology, biochemistry, forensic pathology and medicine, in addition to MST. The utility of PCR- based methods has been widely recognized by academia, regulatory agencies and industry for MST- applications [1-3]. For instance, the EPA MST Guide document states: "This approach (PCR) provides a means to examine targets that are not readily cultivated and may not be in high numbers in the environment, but nevertheless serve as better indicators of fecal sources". In addition, the EPA MST Guide document specifies that for microbial source tracking applications, library independent techniques such as used for the poultry biomarker " rely on the conservation of unique genetic identifiers inherent to a specific fecal microorganism endemic to the members of a single

animal species (the in-group) that are different from the genetic identifiers of the same or different fecal microorganisms in other animals or humans (the out-group)". Stewart et al., [4], (which the Defendants expert witness Samuel Myoda coauthored) stated : "Library independent methods offer many advantages. They have the potential to be considerably cheaper and faster because they do not require the investment in library development. They also have the potential for greater accuracy, since they focus on a specific trait rather than attempting to pattern-match a large number of isolates, some of which may be transient among species."

10. The library-independent PCR MST methods used during the development of the poultry biomarker are widely published in the peer-reviewed literature with an excellent overviews provided in [1-3] and include terminal restriction fragment length polymorphism (T-RFLP), 16S rRNA gene clone libraries, and target-specific PCR-and quantitative-PCR based methods. These techniques are widely-published in literature in MST applications and, specifically, have been used to identify waste-impacted watersheds as a result of humans and cattle [5-8] [9] [10] [11], sea gulls [12], and swine [7, 13] sources. The overall strategy for the development of the poultry biomarker was conducted in a step-wise fashion used by these investigators including:

a. Identify abundant microorganisms within poultry litter and soils to which poultry litter had been applied.

b. Identify what the "targets of interest" using DNA sequencing and analysis and develop PCR assay for these targets. (Targets of interest were identified using clone library analysis and DNA sequencing. In addition, sequence analysis was used to generate primers that would amplify the target DNA sequence using PCR.)

c. Test sensitivity and specificity of potential poultry biomarker using PCR. (The specificity and sensitivity of the potential poultry biomarkers were challenged against a suite of samples that included poultry litter and other major contributors of fecal-waste within the IRW including beef cattle, dairy cattle, swine, duck, geese, waste water treatment plant (WWTP) and septic sewer samples.)

d. Develop and optimize the qPCR protocol for the poultry biomarker. (A quantitative PCR protocol was developed for the poultry biomarker to evaluate the relative ***quantity*** of the poultry biomarker within environmental samples. This was done to evaluate the potential for various fecal sources to contribute significant amounts of the biomarker to the environment, and to analyze the quantity of the biomarker in the environment. In addition, the amplified qPCR DNA sequence could be analyzed using melt curve analysis to distinguish between the poultry biomarker and other closely related organisms that were amplified using the PCR primers. The coupled qPCR assay and melt curve analysis were used to positively identify the presence and relative abundance, if quantifiable, of the poultry biomarker.

11. Poultry litter was used as the source for microbiological evaluation because it is the poultry manure contaminated litter that is directly applied to the environment (i.e. pastures and fields during land application). We verified that the source of the biomarker came from poultry feces, by testing the used litter, unused bedding materials , and poultry feces. These three lines of evidence verify that the biomarker was not present in unused bedding material. The poultry biomarker was detected in the used poultry litter and broiler feces samples from Georgia (average $2.8*10^4$ gene copies/ gram of feces). The unused bedding material did not detect the biomarker.

12. As specified in the EPA MST Guide [2], in order to demonstrate specificity, it is required that it the target DNA sequence (or biomarker) is conserved in the source of interest (i.e. poultry litter) and not found in other sources of pollution (i.e. cattle, human, swine etc.) that may also be significantly impacting a watershed. In fact, the ability to quantify the abundance of the poultry biomarker is one of the key reasons that the qPCR

assay was developed and optimized such that quantification in sources of fecal contamination and within environmental samples collected within the IRW watershed could be conducted. The poultry biomarker has been tested against a variety of non-target fecal samples to determine specificity of the assay. To date, 116 non-target individual and composite fecal samples (representing up to 422 individual samples) collected from beef and dairy cattle, swine, WWTP influent and effluent, septic system samples, geese, ducks, across the United States. In total, eight of these 116 fecal samples gave positive results in the qPCR assay as follows:

- one duck- very low detection in 1 of 13 samples tested representing 58 scats,
- three geese- very low detection in 3 of 25 samples tested representing 70 scats,
- four WWTP influent samples- very low detection in 4 of 27 samples tested, none of which were in the IRW. (It is unknown if sources of the biomarker (i.e. poultry waste) were discharged to the WWTP where the biomarker was detected. None of the corresponding WWTP effluent samples, however, tested positive for the biomarker).

None of these detections, however, were in the IRW, resulting in 100% specificity for non-target samples collected within the IRW and 93% specificity against non-target samples across the US. In addition, the concentration of the biomarker detected in the non-target samples using qPCR was 4-6 orders of magnitude (>10,000-1,000,000 times) lower than the concentration observed in poultry litter. Therefore, the fact that the poultry biomarker was not detected in non-target samples from alternate sources within the IRW and only very small subset of samples had low concentrations in the fecal

material outside the IRW means that detections of the biomarker within the IRW were associated with the presence of poultry litter.

13. In order to evaluate the independent testing of the poultry biomarker by the defendant's expert witness Dr. Samuel Myoda, I reviewed all of the considered materials he and his lab, IEH, provided. Dr. Samuel Myoda's laboratory (IEH), conducted PCR testing using the biomarker primers on fecal samples from gulls, crow, and dogs. None of these samples generated PCR products, verifying that our poultry biomarker is not present in these fecal types. IEH, used PCR testing to evaluate the poultry-specific biomarker rather than the qPCR with melt curve analysis per the standard operating procedures of the poultry biomarker. This significantly undermines the overall interpretation of the IEH data and Dr. Myoda's subsequent conclusions based on these data.

14. The opinions that Dr. Myoda states in section 9.3 of his expert report aren't relevant because the data he provides are not sufficient to support his criticisms regarding the poultry biomarker. There are several lines of evidence that indicate that 1) IEH did not duplicate the methods appropriately, 2) that the biomarker protocol was not implemented with appropriate QA/QC, which invalidates many of their results, and 3) interpretation of much of their own data is incorrect. In order to accurately evaluate the presence of the poultry biomarker, it is important that both qPCR and melt curve analysis be conducted to determine if the product generated in the PCR reaction is in fact the poultry biomarker. Using the qPCR melt curve analysis is the standard for evaluating the

specificity of the biomarker. This analysis has been used extensively by others to distinguish qPCR products generated from closely related microorganisms [14-18]. Coupling qPCR with melt curve analysis provides substantially greater accuracy than evaluating the PCR reaction alone (as Dr. Myoda did), and it is required to distinguish the poultry biomarker.

NO 15. The coupled qPCR assay with melt curve analysis was independently repeated in the laboratory of Dr. Michael Sadowsky to verify that the methods could be reproduced in another lab and successfully optimized using different instrumentation than what was used at the North Wind lab. On the other hand, the independent testing conducted by Dr. Samuel Myoda, and the subsequent basis for many of Dr. Myoda's conclusions in his expert report were based on testing for the poultry biomarker using different methods, namely PCR. While this technique can be used to determine if the biomarker primers will amplify products in a given sample, it does not provide enough information to determine if those products are the poultry biomarker. Dr. Myoda used results of this testing to evaluate specificity of the poultry biomarker and concluded that the PCR assay was not specific to poultry because the primers amplified other organisms, such as *Brevibacterium avium*. While this is true for the PCR method used in the IEH laboratory, it is not true for the optimized qPCR assay with coupled melt curve analysis used for the poultry biomarker and confirmed by Dr. Sadowsky's lab evaluations.

16. There are many examples in the literature where melt curve analysis is used to distinguish base pair differences in large PCR products (157-750 bp products) [14, 15, 17] from closely related organisms.

17. Dr. Myoda and IEH failed to optimize the qPCR protocol and did not conduct quality assurance/quality control on the samples, such as negative DNA extraction controls, and matrix spike samples to validate their results. Their inability to successfully conduct qPCR and melt curve analysis, specifically, was largely a result of 1) not using the appropriate reagent mix in the qPCR reactions, 2) failing to optimize their thermocycling protocol, and 3) failure to conduct a matrix spike sample using the poultry biomarker LA35 plasmid within each sample to compare the subsequent melt curves generated in the sample and generated with the plasmid spiked in the sample, as specified in the our standard operating procedure for the poultry biomarker assay.

18. In contrast, independent testing in Dr. Sadowsky's laboratory verified that when all of these components are included during the development of the qPCR assay, it can be successfully reproduced.

19. IEH provided samples of 13 *Brevibacterium* sp. cultivated from poultry litter collected from the IRW and goose feces collected from Washington State. In addition to the qPCR testing on these IEH samples, North Wind conducted DNA sequencing analysis to verify the exact DNA sequence of these cultivated organisms compared to the DNA sequence of the poultry biomarker. This testing demonstrated that the DNA sequences of the *Brevibacterium* cultures provided by IEH were different than the DNA

sequences of the poultry biomarker, with % sequence similarity ranging from 97%-99%, (Table 1) and therefore, were in fact, not the poultry biomarker. In addition, results of the qPCR with melt curve assay demonstrated distinctly different melt peaks for each of the various *Brevibacterium,* that was distinguishable (> 1°C difference in peak melt temperature) from the poultry biomarker in the spiked samples (Table 1), and the qPCR assay could distinguish between these DNA sequences with only a 5-6 base pair difference in the 531 base pair PCR product.

Table. 1. Summary of results of testing on IEH isolates.

IEH sample ID	MEI identification of the isolated organisms	Isolate sequenced by NWI?	# base pair differences in 531 bp sequence[a]	DNA sequence similarity to poultry biomarker	Amplify by qPCR with NWI poultry biomarker protocol?	Melt peak shift between IEH sample and poultry biomarker
21677-1	Brevibacterium avium	Yes	6	98%	Yes	3.2
21678-1	Brevibacterium avium	Yes	5	98%	No	NA[c]
21679-1	Brevibacterium avium	Yes	6	99%	Yes	1.3
21893-1	Brevibacterium sp.	Yes	6	98%	Yes	1.5
21894-1	Brevibacterium epidermis	Yes	5	99%	Yes	1.4
22644-1	Brevibacterium avium	Yes	5	98%	Yes	2.9 and 1.8[e]
22645-1	Brevibacterium avium	Yes	5	98%	No	NA
22646-1	Brevibacterium sp.	Yes	5	98%	Yes	1.9
22647-1	Brevibacterium avium	Yes	5	98%	Yes	1.5
22648-1	Brevibacterium sp.	Yes	5	98%	Yes	3
22649-1	Brevibacterium avium	Yes	5	98%	Yes	1.1
22650-1	Brevibacterium avium	Yes	5	98%	No	NA
22651-1	Brevibacterium avium	Yes	5	98%	No	NA
21680-1	Corynebacterium ammoniagenes	No	NA[b]	NA[b]	No	NA
21888-1	Pantoea agglomerans	No	NA[b]	NA[b]	Yes	4.1
21889-1	Pantoea agglomerans	No	NA[b]	NA[b]	Yes	4.2
21890-1	Exiguibacterium sp.	No	NA[b]	NA[b]	No	NA

[a] Excludes forward and reverse primer region
[b] NA, Not applicable because the isolates were not analyzed with DNA sequencing
[c] NA, Not applicable because the isolates did not amplify with qPCR
[e] There were two distinct melt curves in this profile indicating that the samples contained two distinct sequences.

20. In addition to the cultivated isolates, Dr. Myoda reported to have "isolated the Plaintiffs' biomarker sequence from unused bedding material, goose flop, sand from a beach frequented by geese, other water fowl samples, and cow hide. Again, however, these conclusions were based on the IEH PCR assay and limited qPCR analysis with a protocol that was not the same as the poultry biomarker protocol. In addition, there were significant issues with the sampling, quality assurance and quality control of this independent sampling effort that must be considered when evaluating the validity of these claims.

21. The Defendants collected two samples of "unused" bedding material from the Simmons poultry barn 25 after the bedding material was spread in the barn, but before any poultry was released in the barn Ex (Myoda deo. 311:20-322:12). IEH observed a positive PCR result (not by use qPCR SOP for these assays) using the biomarker primers on samples that had been placed in media and enriched in the laboratory Ex (Myoda deo. 324:9-325:9). The first divergence in the poultry biomarker protocol is conducting the poultry biomarker assay on a sample that has been enriched in media. Substantial changes in the microbial composition occur during enrichment such that the sample is no longer representative of its original state. In addition, there was a high probability for contamination of these samples in the poultry barn where the "clean" bedding materials were laid down prior to sampling. In order to enrich the *Brevibacterium* in the laboratory, theoretically only one viable cell is needed. Given that poultry litter was demonstrated to contain 10^7-10^9 gene copies/g of litter, it is highly likely that residual used litter containing *Brevibacterium* were present in the poultry house prior to laying

down the clean bedding. There was also potential for contamination during enrichment and extraction in the IEH laboratory, where substantial amounts of *Brevibacterium* isolates were being cultivated in the same room as the DNA extractions and PCR setup used for molecular the IEH analysis, as detailed during Dr. Myoda's deposition Ex (Myoda deo. 40:21-46:6). There is also evidence that the laboratory culture media was periodically contaminated. In addition, IEH were culturing many bacterial isolates in the lab, including ATCC cultures of *Brevibacterium avium*, and *Brevibacterium casei* Ex (Myoda deo. 387:7-389:16), in addition to the isolates that they were cultivating from the "clean" and "dirty" litter, goose feces, Juanita Beach sand, and the cow hide sponge samples Ex (Myoda deo. 41:2-44:12). Under the laboratory setting used for the IEH molecular work, it is critical to do a DNA negative extraction control with this testing because molecular methods are very sensitive and would be easily contaminated (i.e. one cell can be detected by the PCR assay) from the culturing of concentrated and abundant amounts of *Brevibacterium*. No field blank nor DNA extraction negative controls were collected or ran using the PCR protocol that IEH employed. Collectively, this analysis does ***not*** demonstrate that the poultry biomarker was in unused bedding material.

22. IEH also conducted PCR analysis on twelve goose fecal samples collected from Juanita Beach in Washington State (10 samples) and from Green Lake (2 samples) and from four sand samples collected in Juanita Beach in Washington State using the poultry biomarker primers. Similar to the unused bedding material results, no field blank controls were collected during sampling nor DNA extraction controls included in the sample sets analyzed in the IEH laboratory Ex (Myoda deo. 435:18-438:6). In addition,

only two of the goose fecal samples and one sand sample that amplified using the IEH PCR assay was analyzed using qPCR in the considered materials provided by IEH Ex (Myoda deo. 426:8-427:19). Of the two goose fecal samples tested, only one amplified in qPCR, and the melt curve analysis indicated that the PCR products were ***not*** the poultry biomarker. Similarly, the soil sample amplified during the qPCR protocol, but the primer matrix spike was not performed for this sample and the negative qPCR reagent control was contaminated in the qPCR run, invalidating the results, and conclusions based on this assay. Based on these results, it cannot be concluded that Dr. Myoda "isolated" the "biomarker sequence" in these samples.

24. IEH conducted PCR analysis on four cow hide "sponges". The same pervasive errors in sample processing and analysis, however, described for the other samples are relevant for these samples and invalidate these results including failure to analyze field blanks and negative extraction DNA controls Ex (Myoda deo. 449:15-453:14), and contamination in the qPCR assay reagent negative control Ex (Myoda deo. 461:21-465:9). In addition, IEH failed to conduct a matrix spike with the biomarker LA35 plasmid for melt peak comparison.

25. For the *Corynebacterium ammoniagenes* and *Exiguiobacterium* sp., neither the data generated by IEH using their PCR assay nor the North Wind analysis of these samples support Dr. Myoda's claims that our poultry biomarker primers amplified these organisms (Table 1). In addition, *Pantoea agglomerans* were easily distinguished from the poultry biomarker using the melt curve analysis (Table 1). In fact, these data support that the combined poultry biomarker qPCR assay with melt curve analysis was highly specific when challenged against these non-specific cultures.

I declare under penalty of perjury, under the laws of the United States of America, that the foregoing is true and correct.

Executed on the 26th day of May, 2009.

Tamzen Macbeth

Tamzen Macbeth Ph.D., P.E

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